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# Factors Affecting the Outcrossing Rate between Clearfield™ Rice and Red Rice (*Oryza sativa*)

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The commercialization of imazethapyr-resistant (Clearfield™, CL) rice in the southern United States has raised serious concerns about gene flow to red rice, producing imazethapyr-resistant red rice populations. Our objectives were to determine the impact of planting date, CL cultivars, and red rice biotypes on outcrossing rate; and to investigate the relative contribution of flowering time of CL rice and red rice biotypes, together with air temperature and relative humidity (RH), on outcrossing rate. Field experiments were conducted at Stuttgart, Rohwer, and Kibler, AR, from 2005 to 2007, at three or four planting times from mid-April to late May. 'CL161' (inbred cultivar) and 'CLXL8' (hybrid) rice were planted in nine-row plots, with red rice planted in the middle row. Twelve red rice biotypes were used. The flowering of red rice and CL rice, air temperature, and RH were recorded. Red rice seeds were collected at maturity. To estimate outcrossing rate, resistance to imazethapyr was evaluated in subsequent years and confirmed using rice microsatellite markers. CLXL8 rice flowered 2 to 4 d earlier than CL161 rice, and flowering was completed within 1 wk in all plantings. The flowering duration of most red rice biotypes ranged from 4 to 17 d. Flowering synchrony of red rice biotypes and CL rice ranged from 0 to 100% at different plantings. In general, CLXL8 had greater flowering overlap and higher outcrossing rate with red rice than did CL161 rice. The outcrossing rate of red rice biotypes ranged from 0 to 0.21% and 0 to 1.26% with CL161 and CLXL8 rice, respectively. The outcrossing rate differed within each planting date ($P < 0.05$). Outcrossing was generally lower in mid-May and late May than in mid-April and late April planting times. Flowering synchrony and outcrossing rate were not correlated ($r^2 < 0.01$). Outcrossing with CL161 was primarily influenced by red rice biotype. A minimum air temperature of > 24 C in the evening also favors outcrossing with CL161. With CLXL8 rice, outcrossing was most affected by RH. When RH was < 54%, outcrossing was less (0.12%) than when RH was ≥ 54% (0.38%). With CLXL8 rice, a minimum RH of ≥ 54%, from mid-morning to noon, increased outcrossing with red rice. To fully understand the interaction effects of these factors on outcrossing with red rice, controlled experiments are needed.

**Nomenclature:** Imazethapyr; Red rice, *Oryza sativa* L.; Rice, *Oryza sativa* L. ORYSA.

**Key words:** Acetolactate synthase (ALS), biosafety, flowering synchrony, gene flow, herbicide-resistance, imazethapyr-resistant rice, pollen flow, relative humidity.

Weedy red rice, a noxious weed in cultivated rice, can cost producers nearly \$300 $ha^{-1}$ in economic losses (Burgos et al. 2008), mainly because of low harvest efficiency when cultivated rice lodges as a result of red rice infestation (personal observation), contamination of rice grain (Ottis et al. 2005), and rice yield losses from competition with red rice (Shivrain et al. 2009). Limited chemical control options are available for red rice in conventional rice culture (Burgos et al. 2006). The majority of red rice in the southern United States is traditionally controlled by crop rotation between rice and soybean. Recently, herbicide-resistant (HR) CL rice was released, which provides the option for selective control of red rice in rice (Burgos et al. 2008).

Red rice is an annual species belonging to *Oryza sativa*. *Oryza* also includes other annual (Asian wild rice [*O. nivara* Sharma & Shavri], African wild rice [*O. barthii* A. Chev.], and black swamp rice [*O. meridionalis* Ng.]) as well as perennial (brownbeard rice [*O. rufipogon* Griff], long stamen brown rice [*O. longistaminata* A. Chev & Roehr.], and Brazilian wild rice [*O. glumaepatula* Steud.]) species. Most of these species have the potential to hybridize with one another as well as with cultivated rice (Lu and Snow 2005; Noldin et al. 2002). Despite being in a region devoid of other *Oryza* species, red rice populations in the southern United States are genetically and morphologically diverse (Shivrain 2004; Vaughan et al. 2001). This led us to believe that significant variation in outcrossing potential with cultivated rice exists among the weedy populations.

The potential commercialization of transgenic crops (for herbicide resistance, disease and insect resistance, high protein content, virus resistance, salt and drought tolerance) has raised serious biosafety, health, socioeconomic, and ethical concerns (Lu 2008). Among these, the introgression of HR genes via pollen from HR crops to its weedy and wild relatives, which gives rise to HR weedy populations, is the issue that directly impacts weed management. Gene flow from HR crops to weedy relatives can increase fitness of the offspring, leading to the evolution of species with increased weediness. Transgenes conferring traits such as herbicide, disease, and insect resistance, as well as abiotic stress tolerance, have the potential to enhance the fitness of wild relatives (Snow et al. 1998; Stewart et al. 1997). However, in the majority of cases reported thus far, outcrosses demonstrated reduced or equal fitness relative to their parents (Arriola and Ellstrand 1997; Oard et al. 2000; Snow et al. 1998; Zhang et al. 2003).

The risk of outcrossing is difficult to accurately predict because many factors can alter the detectable outcrossing rate. In general, gene flow from cultivated rice to red rice biotypes depends on (a) flowering time of rice and red rice, (b) genetic compatibility between rice and red rice, (c) pollen load, (d) floral morphology of both species, and (e) environmental conditions. Studies have shown that gene flow can occur even in self-pollinated crops if the compatible species present are sympatrically distributed and have synchronous flowering (Lu et al. 2003; Vaughan 1994). In China, brownbeard rice and

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two cultivars (a late-maturing local variety and an improved Minghui-63) had a significant overlap in pollination time, which resulted in outcrossing (Lu et al. 2003). Red rice usually has a protracted flowering period, creating conditions favorable for overlapping flowering times with cultivated rice (Lu and Snow 2005), which varies significantly depending on geographical origin (Lu et al. 2003; Shivrain 2004). The overlap in flowering period of transgenic rice (pollen donor) and conventional rice or red rice (both pollen acceptors) has been directly correlated with outcrossing rate (Messeguer et al. 2004).

Rice and its weedy or wild relatives are considered low-risk species in terms of outcrossing because of their self-pollinating nature (Stewart et al. 1997), but gene flow (< 1%) from cultivated rice to weedy rice has been reported (Chen et al. 2004; Messeguer et al. 2004; Shivrain et al. 2006, 2007). So far, 52% is the highest reported outcrossing frequency, which occurred between red rice and the rice cultivar 'Nortai' in Louisiana (Langevin et al. 1990). In China, an 18% outcrossing rate was documented between transgenic rice and wild rice (Wang et al. 2006). Information on individual outcrossing potential and variation in flowering times aid in the evaluation of the potential for hybridization (Lu and Snow 2005). It has been documented that the outcrossing rate between Indica cultivars and wild rice is greater than between Japonica cultivars and wild rice (Oka 1988). In Arkansas, differences in outcrossing rate between CL161 (0.008) and 'CL121' (0.003%), both Japonica cultivars, with red rice has been documented (Shivrain et al. 2006).

CL rice hybrids are commercialized in the southern United States. Hybrid rice produces higher yield than traditional rice cultivars, which holds true for CL hybrids (Walker et al. 2008). Improved red rice control in CL rice production system has resulted in increased rice yields; thus, areas planted to CL rice cultivars are expected to expand. To mitigate outcrossing and sustain the efficacy of CL technology, investigating the plant and environmental factors influencing outcrossing is important. How much the air temperature and RH affect outcrossing between cultivated rice and red rice is not known, although the importance of these factors on successful hybridization between rice cultivars has been established (Satake and Yoshida 1978). Thus, our objectives were to (1) determine the impact of planting date, CL cultivars, and red rice biotypes on the outcrossing rate and (2) evaluate the relative influence of flowering time of CL rice and red rice biotypes, air temperature, and RH on outcrossing rate.

## Materials and Methods

**Flowering and Screening for Outcrosses.** To determine the flowering time and synchronization in flowering of red rice biotypes and cultivated rice, experiments were conducted at the Rice Research and Extension Center (RREC), Stuttgart, AR, and the Southeast Research and Extension Center (SREC), Rohwer, AR. Stuttgart and Rohwer represent the Delta and Grand Prairie agro-ecological zones in Arkansas, respectively. The experimental design was a split-split-plot with three and four replications at Stuttgart and Rohwer, respectively. The main plot factor was planting date, the subplot-factor was CL rice cultivar, and the sub-subplot factor was red rice biotype. There were four plantings at 2-wk intervals at Stuttgart in both years, but because of weather complications, only three plantings in 2005 and two plantings in 2006 were done at Rohwer (Table 1). The CL rice cultivars used were CL161 and CLXL8, which were imazethapyr-resistant conventional and hybrid rice, respectively. Twelve red rice biotypes were used, representing the range of plant heights, maturity periods, and hull colors of the weedy rice plants in Arkansas. These were collected in the summers of 2002 and 2003 from 10 counties (Shivrain 2004) where nearly 40% of rice in Arkansas is grown (Wilson and Branson 2007). Twenty-five seeds of each red rice biotype were planted in the middle row of plots, 5 m long, with 18-cm-wide rows, flanked by four rows of CL rice cultivars on both sides. CL161 and CLXL8 were planted at 104 and 34 kg ha$^{-1}$, respectively.

Table 1. Planting dates of experiments at the Rice Research and Extension Center, Stuttgart, and the Southeast Research and Extension Center, Rohwer, AR, 2005 and 2006.

| | Stuttgart | | Rohwer [a] | |
|---|---|---|---|---|
| Planting date | 2005 | 2006 | 2005 | 2006 |
| Mid-April | April 16 | April 17 | — | — |
| Late April | April 27 | April 26 | April 28 | — |
| Mid-May | May 13 | May 15 | May 12 | May 23 |
| Late May | May 26 | May 25 | May 26 | June 07 |

[a] Only three plantings in 2005 and two plantings in 2006 were possible at Rohwer because of weather-related complications.

Data on flowering of red rice and CL rice were collected every other day at Stuttgart from the inception of flowering. At grain filling, red rice panicles were enclosed in Delnet® bags[1] to collect seeds. Air temperature (minimum, maximum, average) and RH (minimum, maximum, average) were recorded every 30 min from planting to harvest using weather data loggers[2] placed in the bays. At maturity, red rice seeds were harvested and stored at room temperature until planting in subsequent years. A subsample of approximately 3,000 red rice seeds from each plot was planted at the SREC and at the Vegetable Research Substation, Kibler, AR, in 2006 and 2007, respectively, in 12-row, 12-m-long plots. CL161 and CLXL8 rice and the 12 red rice biotypes from the original population were planted as checks. Seedlings were counted at the two-leaf stage. Imazethapyr[3] at 0.14 kg ai ha$^{-1}$ + nonionic surfactant (0.25% v/v) was applied twice at a 7-d interval starting at the two-leaf stage of rice. Six weeks after the initial application of imazethapyr, leaf tissues were collected from the survivors and from the parents (CL161, CLXL8, and red rice biotypes) for DNA analysis to confirm outcrossing.

**Confirmation of Outcrosses.** DNA from imazethapyr survivors was extracted using the protocol of Xin et al. (2003). Outcrosses were confirmed following the protocol used by Shivrain et al. (2008). DNA from survivors was used as a template in PCR, using rice simple sequence repeat (SSR) primer pairs RM234 and RM253. Amplifications of SSR fragments were performed in MJ Research Tetrad Thermal Cyclers[4] with the following temperature profile for RM234: one cycle of 94 C (2 min); 34 cycles of 94 C (30 s), 55 C (30 s), and 72 C (1 min); a final cycle of 72 C (5 min); and incubation at 4 C. For RM253, the temperature profile was one cycle of 94 C (2 min); 34 cycles of 94 C (30 s), 51 C (30 s), and 72 C (1 min); a final cycle of 72 C (5 min); and incubation at 4 C until DNA fragment analysis. Amplified products were separated by capillary electrophoresis on an ABI prism 3730 DNA Analyzer[5] according to the manufacturer's

Table 2. Size of alleles amplified in outcrosses between 12 red rice biotypes and Clearfield™ rice cultivars, CL161 and CLXL8, using microsatellite markers RM234 and RM253.

| | Allele size | | | |
|---|---|---|---|---|
| | CL161 | | CLXL8 | |
| Red rice | RM234 | RM253 | RM234 | RM253 |
| | bp | | | |
| Ash-1 | 135/144 | 130/139 | 135/144 | 132/139 |
| Chi-4 | 135/155 | 130/139 | 135/155 | 130/139 |
| Chi-5 | 135/155 | 130/141 | 133/155 | 130/141 |
| Cla-3 | 135/155 | 130/139 | 135/155 | 132/139 |
| Dre-2 | 135/144 | 120/130 | 135/144 | 120/132 |
| Gre-5 | 135/153 | 130/136 | 133/153 | 130/136 |
| Lon-1 | 135/155 | 130/141 | 135/155 | 130/141 |
| Phi-1 | 135/155 | 130/141 | 135/144 | 130/139 |
| Poi-1 | 135/144 | 120/130 | 135/144 | 120/130 |
| Poi-4 | 135/153 | 120/130 | 133/155 | 120/130 |
| Pop-1 | 135/155 | 130/141 | 135/155 | 130/141 |
| Ran-5 | 135/155 | 130/139 | 135/155 | 130/139 |

instructions. Genemapper[6] was used to identify amplified PCR products obtained at each locus, followed by manual binning where necessary. The size of specific amplified fragments for each red rice biotype and CL rice are presented in Table 2.

Flowering synchronization of red rice and CL rice, air temperature, and RH were recorded. The flowering synchronization period was calculated based on the number of days when red rice and CL rice flowered simultaneously. The estimation of flowering synchronization was based on the flowering time of CL rice because gene flow to red rice depends on the availability of CL rice pollen. Flowering overlap was indicated 100% when the onset and completion of flowering of CL rice fell within the flowering period of red rice and 0% when there was no overlap in flowering period of CL rice and red rice (Shivrain et al. 2008). Air temperature and RH data were divided into time periods within the day (1,000 to 1,300 h; 1,800 to 2,200 h, and 0 to 2,400 h), which could potentially impact pollen tube development and seed set (Moldenhauer and Gibbons 2002).

**Statistical Analysis.** The outcrossing rate between red rice biotypes and CL rice types was estimated based on the number of confirmed outcrosses. To compensate for the nonnormalized outcrossing rate, data were transformed by calculating the logarithm of the outcrossing rate. However, ANOVA and means separation were similar for transformed and nontransformed data; thus, nontransformed outcrossing data were analyzed. Data from both locations and years were combined after confirmation of homogeneity of variance by Bartlett's test (Steel and Torrie 1980). Data for outcrossing were subjected to the GLM Procedure in SAS[7] to test all possible interactions of locations, planting dates, CL rice cultivars, and red rice biotypes. Type III statistics were used to test all possible fixed effects or interactions among the fixed effects, and the least significant difference at $P \leq 0.05$ was used for means separation using the Tukey's HSD test.

Correlation analysis was performed between synchrony in flowering and outcrossing rate of red rice biotypes. In addition, the impact of red rice biotype, air temperature, and RH on outcrossing was analyzed. Using all these data, various linear, polynomial, sigmoidal, nonlinear, and neural net models were tested using JMP[8] software to estimate the outcrossing rate. None of these models could describe the sources of variation in the data. However, recursive partitioning, which describes the relationship between the X and Y values by creating a tree of partitions, was able to describe the relationship between the aforementioned variables and outcrossing rate. Partitioning finds a set of binary splits or groupings of X values that best predict a Y value (Anonymous 2008) by exhaustively searching all possible splits or groupings. These splits (or partitions) of the data are done recursively, forming a tree of decision rules, until the desired fit is reached.

## Results and Discussion

**Flowering Time and Synchrony in Flowering of Red Rice and CL Rice.** There was a 2- to 3-d variation in the initiation of flowering of red rice and CL rice both years, which affected their overlap in flowering period with CL rice (Figure 1 and 2). Because flowering was recorded every other day, the data collected are an approximation of the overlap in the flowering time of red rice biotypes and CL rice. In general, CL rice and red rice biotypes planted at an earlier date took more days to flower than those planted later (data not shown). The onset of flowering of red rice biotypes ranged from 78 to 128, 76 to 113, 70 to 116, and 64 to 114 days after planting (DAP) in mid-April, late April, mid-May, and late May plantings, respectively. CLXL8 flowered 2 to 4 d earlier than CL161 rice on average, although flowering was completed within a week (88 to 100 DAP) in all plantings in both CL cultivars. Red rice in general has a protracted flowering duration of 4 to 17 d, whereas rice cultivars finished flowering within a week (Shivrain et al. 2008).

In general, the overlap in flowering of red rice biotypes and CL rice was similar in both years (Figure 1 and 2). However, the flowering overlap in the majority of red rice biotypes with both CL rice cultivars was greater during mid-May planting in 2006 than at the same period in 2005. In at least one planting date, the majority of red rice biotypes had $>$ 60% flowering synchrony with CL161, except Gre-5 and Poi-4 in 2005 and 2006, and Cla-3 in 2006 (Figure 1). Gre-5, the earliest to flower (70 DAP), had almost no overlap in flowering with CL161. Likewise, Poi-4, which flowered the latest (100 DAP) had minimal overlap in flowering with CL161 in both years. In general, the flowering synchrony of red rice biotypes and CL161 was less in the mid-May and late May plantings than in the mid-April and late April plantings. In general, Chi-5, Poi-1, Pop-1, and Ran-5 had $>$ 50% flowering synchrony with CL161 at all planting dates except for the mid-May planting in both years. Cla-3, Dre-2, and Phi-1 did not synchronize in flowering with CL161 in the mid-May planting during 2005, but some flowering synchrony occurred in 2006. Poi-4 had minimal flowering overlap with CL161 (2 to 10%) in all four plantings in 2005, but none in the mid-May and late May planting in 2006.

Because the flowering duration of CLXL8 was 2 to 3 d longer than that of CL161, its flowering synchrony with all the red rice biotypes was higher. Flowering synchronization of Cla-3, Dre-2, and Phi-1 with CLXL8 was also minimal or none at all (Figure 2), as was observed with CL161. The degree of overlap in flowering varied by year within the same planting date, primarily due to variability in climatic conditions. These experiments demonstrated that flowering time of red rice biotypes differs greatly and gene flow rates are

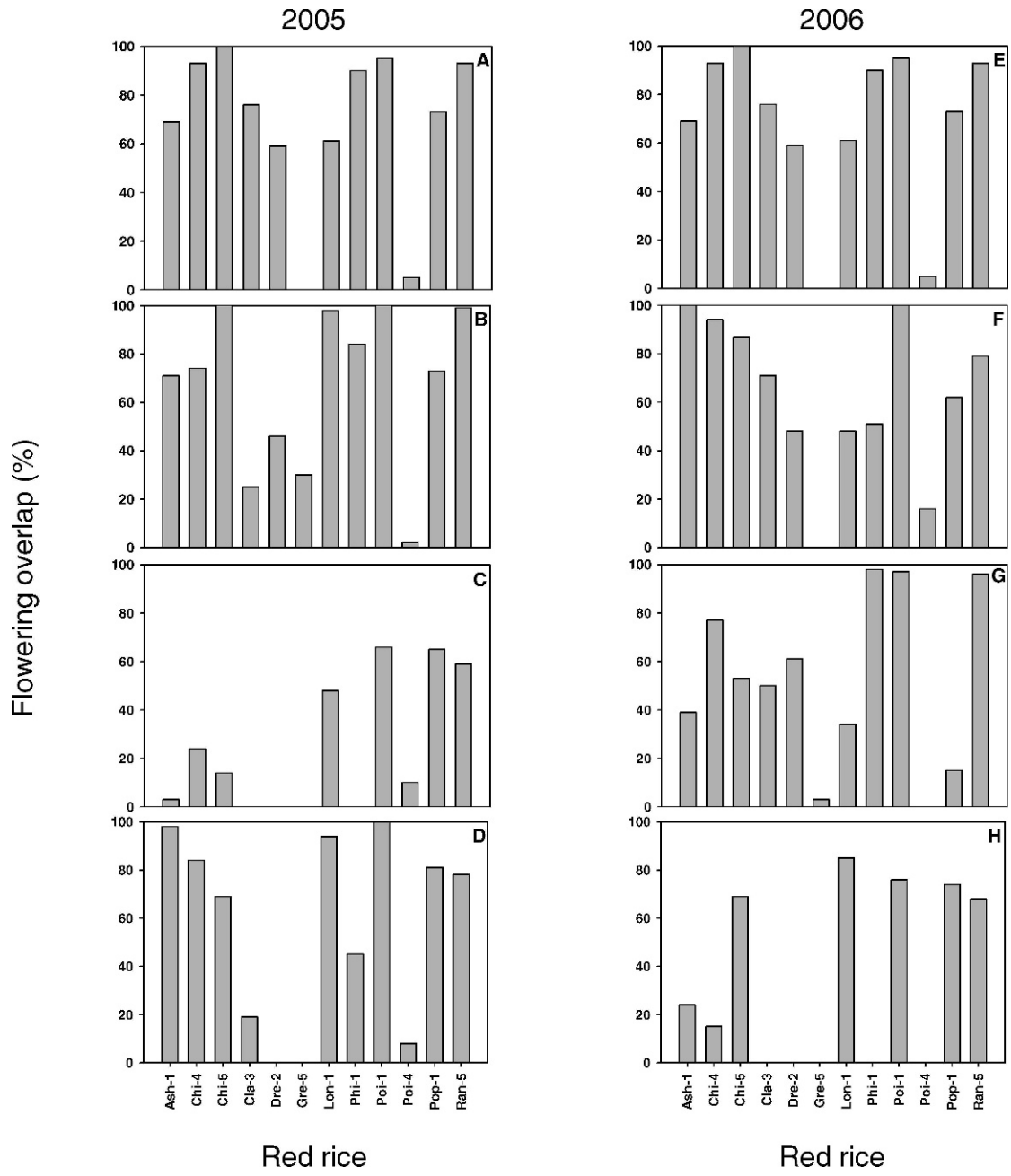


Figure 1. The approximate overlap percentage in flowering of 12 red rice biotypes and CL161 rice in mid-April (A and E), late April (B and F), mid-May (C and G), and late May (D and H). Data were averaged over two locations.

also expected to vary by red rice biotype. Some red rice biotypes can flower as early as 55 DAP (Shivrain et al. 2009) relative to most of the rice cultivars in the southern United States, which initiate flowering at 85 to 95 DAP. These early-flowering biotypes are less of a risk from an HR gene transfer perspective, but they could reduce rice yield significantly (Shivrain et al. 2009) and replenish the soil seed bank when seeds shatter. Overall, the majority of red rice biotypes used in this study, which represent the types present in Arkansas, had $>$ 40% overlap in flowering time with both inbred and hybrid CL rice in at least one planting date.

**Outcrossing Rate.** The location effect on outcrossing was not significant; thus, data were pooled over locations. Significant differences in outcrossing rate with red rice occurred between the two rice cultivars. The year effect on outcrossing of CL161 with red rice was not significant; thus, CL161 data were pooled over years. The outcrossing rate of CLXL8 with red rice was affected by year, so data were analyzed by year. For both CL rice types, the interaction between planting date and red rice biotype on outcrossing rate was significant ($P < 0.01$).

*CL161 and Red Rice Biotypes.* Averaged over 2 yr, the outcrossing rate between CL161 and red rice biotypes ranged from 0.01 to 0.17, 0.02 to 0.21, 0.01 to 0.16, and 0 to 0.21% in the mid-April, late April, mid-May, and late May plantings, respectively (Table 3). The outcrossing rate within

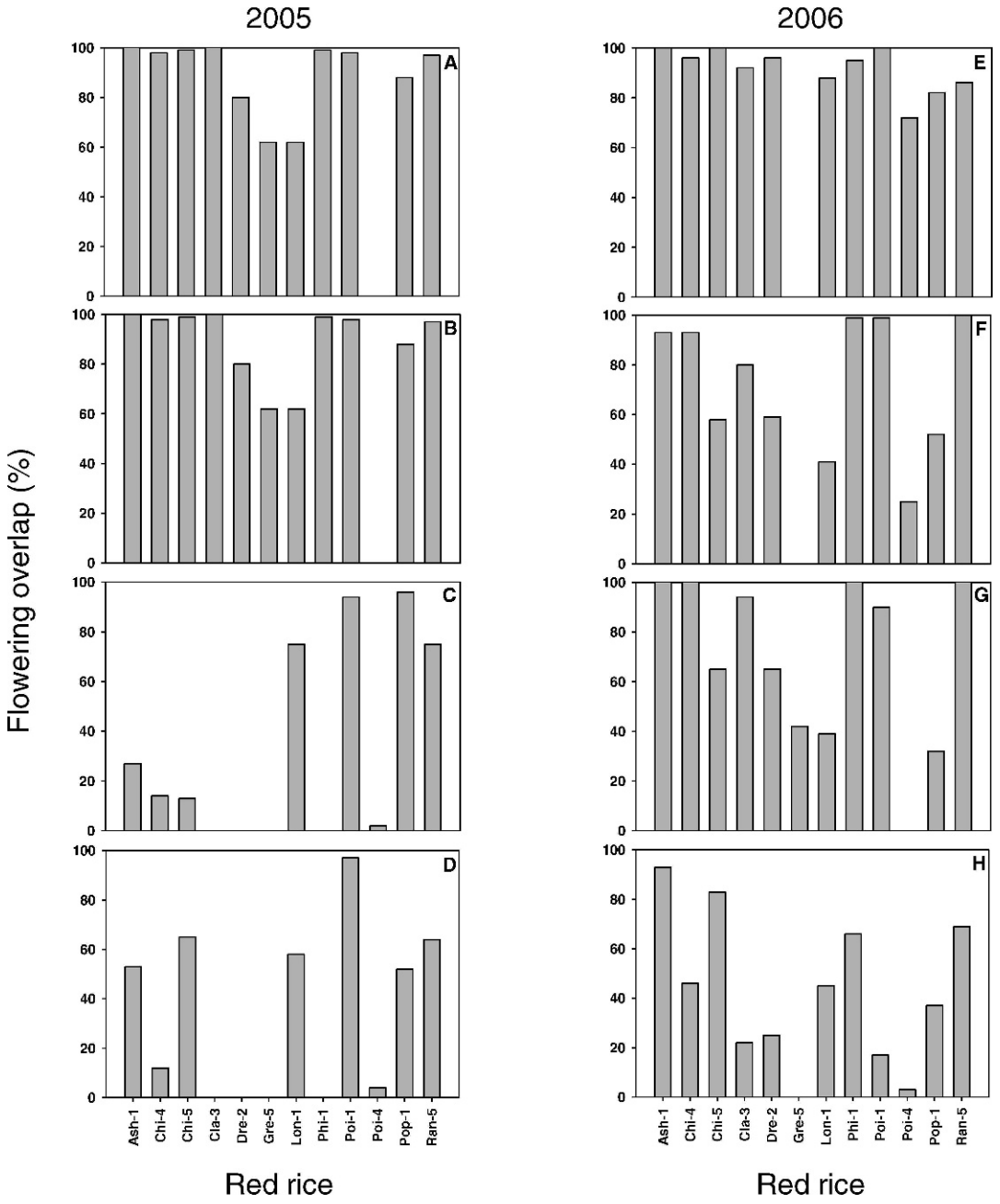


Figure 2. The approximate overlap percentage in flowering of 12 red rice biotypes and CLXL8 rice in mid-April (A and E), late April (B and F), mid-May (C and G), and late May (D and H). Data were averaged over two locations.

each planting time differed by red rice biotype and also differed between planting times. Outcrossing with Cla-3 (0.04 to 0.08%), Phi-1 (0.02 to 0.05%), and Poi-4 (0 to 0.03%) was not affected by planting time. Gre-5, the earliest flowering red rice type, had greater outcrossing in the mid-April planting (0.16%) than in the later plantings (0.02 to −0.04%). Poi-4, the latest flowering red rice type, had consistently low outcrossing rate with CL161, except in the mid-May planting where it registered its highest outcrossing rate. Chi-4, Chi-5, and Poi-1 had greater outcrossing rates when they flowered around 100 DAP in mid-April and 95 DAP in the late April planting dates, compared with later plantings (data not shown). The highest outcrossing rate with Dre-2 (0.13%) occurred in the mid-May planting compared with the April and late May plantings. The highest outcrossing rates in the late April planting were with Ash-1 (0.2%), Chi-4 (0.21%), Chi-5 (0.12%), Poi-1 (0.1%), and Ran-5 (0.17%). In the late May planting, Ash-1 had the greatest outcrossing (0.21%) among all red rice biotypes; the rest had 0 to 0.07%. Overall, CL161 had the highest outcrossing rates with Chi-4 (up to 0.21%), Gre-5 (up to 0.16%), and Ash-1 (up to 0.21%). Averaged over planting dates, Ash-1 produced the most outcrosses with CL161. The outcrossing rates were generally lower in the mid-May and late May plantings than in the mid-April and late April plantings. The spread in outcrossing rates across planting dates and across biotypes demonstrate that there are critical times when the risk of gene flow is relatively high or, conversely, nonexistent. The high risk times differ by red rice biotype, so it is critical to determine when this would occur in

Table 3. Outcrossing rate (%) of 12 red rice biotypes and Clearfield™ rice, CL161 at the Rice Research and Extension Center, Stuttgart, AR, and the Southeast Research and Extension Center, Rohwer, AR, 2005 and 2006.[a]

| Red rice | Outcrossing rate | | | |
|---|---|---|---|---|
| | Mid-April | Late April | Mid-May | Late May |
| | % | | | |
| Ash-1 | 0.14 | 0.20 | 0.16 | 0.21 |
| Chi-4 | 0.17 | 0.21 | 0.02 | 0.06 |
| Chi-5 | 0.10 | 0.12 | 0.01 | 0.00 |
| Cla-3 | 0.07 | 0.04 | 0.08 | 0.04 |
| Dre-2 | 0.04 | 0.04 | 0.13 | 0.01 |
| Gre-5 | 0.16 | 0.04 | 0.02 | 0.02 |
| Lon-1 | 0.04 | 0.03 | 0.08 | 0.02 |
| Phi-1 | 0.04 | 0.05 | 0.04 | 0.02 |
| Poi-1 | 0.07 | 0.10 | 0.01 | 0.05 |
| Poi-4 | 0.01 | 0.02 | 0.03 | 0.00 |
| Pop-1 | 0.05 | 0.02 | 0.08 | 0.02 |
| Ran-5 | 0.07 | 0.17 | 0.09 | 0.07 |
| LSD $_{(0.05)}$ | | | | |
| within planting | | 0.01 | | |
| across plantings | | 0.05 | | |

[a] Data were pooled over year and locations. There were only three and two planting dates at Rohwer in 2005 and 2006, respectively.

certain biotypes, and which ones would tend to have higher risk than others. This variability in outcrossing rates and peaks of outcrossing were mirrored by the data from CLXL8.

*CLXL8 and Red Rice Biotypes*. The outcrossing rate between CLXL8 and red rice biotypes ranged from 0.0 to 1.26, 0 to 0.40, 0.0 to 1.26, and 0.0 to 0.2% in the mid-April, late April, mid-May, and late May planting dates, respectively, in 2005 (Table 4). CLXL8 tended to have a higher outcrossing rate with red rice than did CL161; however, CLXL8 did not outcross with some red rice biotypes (Chi-4, Gre-5, Lon-1, Poi-4, Pop-1), in some planting dates with which CL161 outcrossed. This difference between the two CL rice types could be due to their differential genetic compatibility with red rice (Shivrain et al. 2008). Hybrid rice has some Indica traits: i.e., tall, high tiller production, and grain shattering. Since red rice is an Indica type rice, it would be genetically closer to hybrid rice than the inbred cultivar, which is a Japonica type.

The outcrossing rate in 2006 was lower than that in 2005, ranging from 0.02 to 0.46, 0.0 to 0.80, 0.02 to 0.36, and 0.0 to 0.23% in mid-April, late April, mid-May, and late May, respectively. The outcrossing rate of CLXL8 with Lon-1, a late-flowering red rice biotype (100 DAP), was low in both years at 0 to 0.02%. Poi-4, another late-flowering biotype, which showed zero to minimal outcrossing rate with CL161, had even less outcrossing with hybrid rice. Poi-4 had no detectable outcrossing in 2005, except in the May 15 planting, and showed consistently low outcrossing rates in 2006. The red rice biotypes differed in outcrossing rate with CLXL8 within each planting date as was observed with CL161. In 2005, the highest outcrossing rates in the mid-April planting were with Ash-1 (0.34%), Chi-5 (0.29%), Cla-3 (0.46%), Dre-2 (1.17%), and Gre-5 (1.26%). The lowest average outcrossing rate among planting dates was in the late May planting. Among all the accessions and planting dates, the highest outcrossing rate with CLXL8 (1.26%) was observed in two red rice biotypes (Gre-5, Ran-5) in 2005. In 2006, the outcrossing rates of Cla-3 (0.03 to 0.12%), Lon-1 (0 to 0.1%), Poi-1 (0.08 to 0.18%), Poi-4 (0 to 0.06%), and Pop-1 (0.06 to 0.12%) were not affected by planting dates. Chi-4, Chi-5, and Ran-5 had greater outcrossing with CLXL8 in the mid-April and late April planting dates than in the mid-May and late May plantings in 2006. The highest outcrossing rate of CLXL8 with Dre-2 and Gre-5 occurred in the mid-May planting. In general, in 2006, outcrossing was lower in the mid-May and late May plantings than in the mid-April and late April planting dates, as was observed with CL161. Among all planting dates and red rice accessions in 2006, the highest outcrossing rate with CLXL8 (0.8%) was observed with Ash-1 in the late April planting; this was observed with Gre-5 at 1.26% in the mid-April planting and Ran-5 at 1.26% in the mid-May planting in 2005. Red rice biotypes that would pose the highest risk of outcrossing would vary from year to year as this data showed. The highest risks of outcrossing in 2005 were with Ran-5, Gre-5, Dre-2, Ash-1, and Cla-3; whereas in 2006 it was with Ash-1 and Dre-2. Most consistently, Ash-1 and Dre-2 posed the highest risk of

Table 4. Outcrossing rate (%) between 12 red rice biotypes and Clearfield™ rice CLXL8 planted at the Rice Research and Extension Center, Stuttgart, AR, and the Southeast Research and Extension Center, Rohwer, AR, 2005 and 2006.[a]

| Red rice | Outcrossing rate | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2005 | | | | 2006 | | | |
| | Mid-April | Late April | Mid-May | Late May | Mid-April | Late April | Mid-May | Late May |
| | % | | | | | | | |
| Ash-1 | 0.34 | 0.22 | 0.42 | 0.10 | 0.16 | 0.80 | 0.18 | 0.23 |
| Chi-4 | 0.00 | 0.00 | 0.34 | 0.03 | 0.46 | 0.47 | 0.15 | 0.06 |
| Chi-5 | 0.29 | 0.12 | 0.15 | 0.04 | 0.22 | 0.14 | 0.03 | 0.07 |
| Cla-3 | 0.46 | 0.18 | 0.02 | 0.20 | 0.10 | 0.08 | 0.03 | 0.12 |
| Dre-2 | 1.17 | 0.18 | 0.01 | 0.02 | 0.10 | 0.20 | 0.36 | 0.20 |
| Gre-5 | 1.26 | 0.25 | 0.00 | 0.00 | 0.02 | 0.05 | 0.24 | 0.00 |
| Lon-1 | 0.02 | 0.00 | 0.06 | 0.00 | 0.10 | 0.00 | 0.02 | 0.00 |
| Phi-1 | 0.08 | 0.34 | 0.03 | 0.06 | 0.17 | 0.28 | 0.08 | 0.02 |
| Poi-1 | 0.02 | 0.04 | 0.29 | 0.10 | 0.08 | 0.14 | 0.12 | 0.18 |
| Poi-4 | 0.00 | 0.00 | 0.20 | 0.00 | 0.06 | 0.01 | 0.02 | 0.00 |
| Pop-1 | 0.07 | 0.05 | 0.51 | 0.00 | 0.06 | 0.06 | 0.12 | 0.09 |
| Ran-5 | 0.05 | 0.40 | 1.26 | 0.06 | 0.20 | 0.22 | 0.07 | 0.08 |
| LSD $_{(0.05)}$ | | | | | | | | |
| within planting | | 0.04 | | | | 0.06 | | |
| across plantings | | 0.08 | | | | 0.12 | | |

[a] Data were analyzed by year, pooled over locations. There were only three and two planting dates, respectively, at Rohwer in 2005 and 2006.

outcrossing across planting dates and years. Poi-4 had consistently the lowest outcrossing rate, as was observed with CL161. This was not surprising considering that flowering synchrony of Poi-4 and the rice cultivars was nil.

**Synchrony in Flowering and Outcrossing Rate.** Gene flow between two species is affected by their proximity as well as synchrony in flowering time (Gealy et al. 2003). However, there was no correlation ($r^2 < 0.1$) between the overlap in flowering time of red rice and CL rice and outcrossing rate in these experiments. The flowering synchrony between red rice biotypes and CL rice ranged from 0 to 100% (Figure 1 and 2). There were cases where some outcrossing occurred when there was no apparent overlap in flowering time (i.e., CL161 and Gre-5) at three planting dates. This could be due to the late-flowering florets on red rice spikes. Unlike cultivated rice (Moldenhauer and Gibbons 2002), the opening of florets on red rice spikes is highly variable. Red rice also produces late tillers, which flower much later than the older ones. These late events could occur beyond the time when the duration of red rice flowering was evaluated. In this experiment we did not document the tillering pattern of red rice or CL rice; however, in related experiments, we have observed that red rice produces tillers for an extended period of time compared with CL rice (Shivrain et al., unpublished data). Some florets on a rice spike may have flowered and transferred pollen to red rice the day before data collection, and flowering was thus recorded to have occurred a day later. Finally, it might not take much flowering synchronization to affect a high rate of cross-pollination. On the other hand, Chi-5, which had at least 50% overlap in flowering with CL161 in the late May planting, did not outcross with CL rice. Chi-5 also had the least seed set (49%) in manual crosses with CL161 (Shivrain et al. 2008), which indicates low genetic compatibility with CL161. In general, however, outcrossing occurred when there was a flowering overlap.

**Relative Impacts of Plant and Environmental Factors on Outcrossing Rate.** To evaluate the relative contribution of red rice biotype, flowering synchrony, air temperature, and RH on outcrossing, data were subjected to recursive partitioning. Instead of planting time, temperature and RH during the flowering period of cultivated and weedy rice were included in the analysis. Data were analyzed separately for the CL rice types because of high variation in the outcrossing pattern between the two types. Data partitioning was performed up to the point that further partitioning no longer improved the $r^2$ value. For CL161 rice, the greatest $r^2$ (0.60) was achieved after partitioning the data 10 times, whereas for CLXL8 rice, the greatest $r^2$ (0.58) data were partitioned 12 times. The $r^2$ values were confirmed with K-fold–cross-validation, which randomly assigns all the data to one of $k$ groups, then calculates the error for each point using means or rates estimated from all the groups, except the group to which the point belongs.

The overall mean outcrossing rate for CL161 rice was 0.07% over 2 yr and two locations (Figure 3). The primary factor that affected the outcrossing rate with CL161 rice was red rice biotype. The red rice biotypes separated into two groups based on their average outcrossing rate. The first group, composed of nine red rice biotypes, had nearly half the outcrossing rate (0.05%) of the second group (0.13%). The primary factor that affected the outcrossing rate of the first group of nine red rice biotypes was the minimum temperature at 1,800 to 2,200 h; temperatures lower than 24 C resulted in an average outcrossing rate of 0.04%, which increased to 0.10% at higher temperatures. The second factor that affected the outcrossing rate of the first group was RH at 1,000 to 1,300 h; RH < 74% resulted in an average outcrossing rate of 0.04%, which increased to 0.20% at higher humidity. Flowering synchrony and the maximum temperature during 1,000 to 1,300 h were the other factors that influenced the outcrossing rate of the first group of red rice biotypes. For the second group (Ash-1, Chi-4, and Ran-5), which had a high outcrossing rate (0.13%), the primary factor affecting outcrossing rate was the average temperature at 1,800 to 2,200 h, followed by the red rice biotype. The outcrossing rate in these three red rice biotypes changed significantly with temperature (Figure 3).

The outcrossing rate for CLXL8 rice was 0.23% averaged over 2 yr, 12 red rice biotypes, and two locations (Figure 4). The primary factor that impacted the outcrossing rate of these red rice biotypes with CLXL8 rice was the minimum RH at 1,000 to 1,300 h. If the minimum RH stayed below 54%, then the outcrossing rate was almost one-third of what it was when the RH was > 54%. During the rice flowering season in Arkansas, RH is generally > 60%, which favors cross-pollination. In tropical regions where RH is normally high, it can then be expected that outcrossing rates of hybrid rice with weedy rice would be higher than what was observed in the experiments reported here. The second factor that influenced the outcrossing rate with CLXL8 rice was the temperature at 1,000 to 1,300 h. A minimum temperature of < 24 C caused greater outcrossing rates (0.58%) than did temperatures above 24 C (0.25%). When the minimum temperature was higher than 24 C in the 1,000- to 1,300-h period, the second factor that influenced the outcrossing rate was red rice biotype. As observed with CL161 rice, the red rice biotypes fell into two groups based on their outcrossing rate with CLXL8. The first group of red rice biotypes had a lower outcrossing rate (0.16%) than the second group (0.54%).

Previous studies have shown that the flowering of rice is influenced greatly by temperature and RH during the flowering period (Satake and Yoshida 1978). In general, flowering is the developmental stage most susceptible to temperature variations (Jagadish et al. 2007), which ultimately affects seed set. The heat tolerance of pollen varies significantly among different rice cultivars (Matsui et al. 1997). In general, Indica rice tolerates higher temperatures than Japonica types (Satake and Yoshida 1978). Gene flow from cultivated rice to red rice at high temperature could be limited more by the life span of the pollen (affected by environmental conditions) than the receptivity of the red rice stigma. RH during flowering of rice impacts pollen viability and seed set (Matsui et al. 1999). In field conditions, temperature and RH are interdependent and so are their impacts on pollen viability and seed set.

In summary, the most important factor that influenced outcrossing rate with CL161 rice was the red rice biotype. This comprises the genetic compatibility with CL161 rice and phenological differences between red rice biotypes. Three red rice biotypes (Ash-1, Chi-4, and Ran-5) have outcrossing rates nearly three times higher than the other nine red rice biotypes. The second most important factor was the minimum and average temperature at 1,800 to 2,200 h. On the other hand,

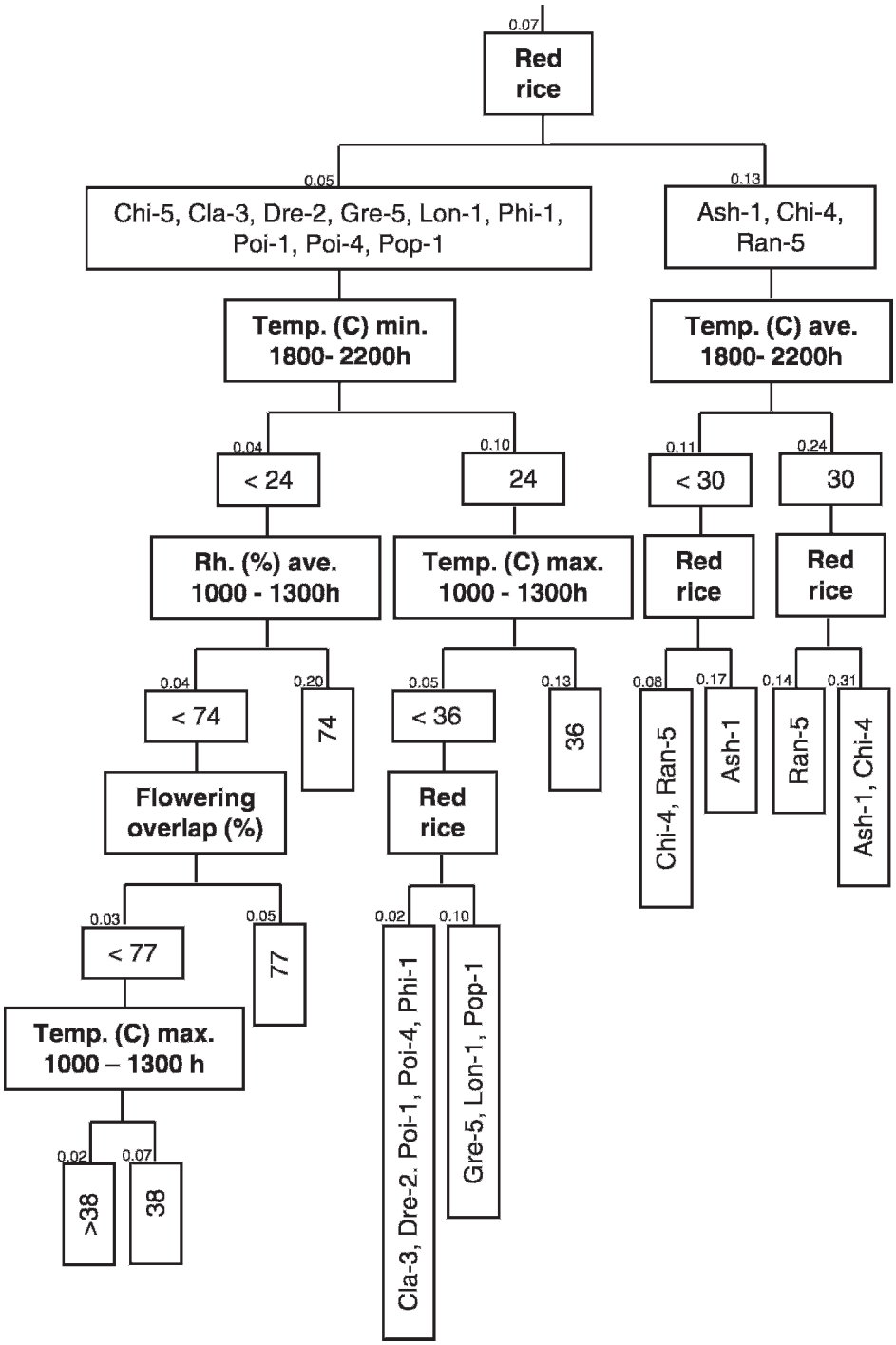


Figure 3. Partition flow diagram for the outcrossing rate of 12 red rice biotypes with CL161 rice. The relative importance of factors (in bold) decreases from top to bottom. Values at the nodes describe the average outcrossing rate percentage of the subset of data that is evaluated at that partitioning. Abbreviations: Temp., air temperature; Rh., relative humidity; min., minimum; max., maximum; ave., average.

for CLXL8 rice, RH was the most important factor for successful cross- pollination. The hybrid and inbred lines may have different pollen characteristics. With hybrid rice, red rice biotype was less important than RH because there was less genetic compatibility barrier between hybrid rice and red rice than with inbred rice and red rice. Other factors that influence gene flow include pollen load, floral morphology, and pollen viability (Gealy 2005; Virmani and Athwal 1973); however, we did not measure these in this experiment.

In this study, we determined that CL161 had less outcrossing rate (0.21%) with red rice compared with CLXL8 (1.26%), averaged over red rice biotypes. The outcrossing patterns with different red rice biotypes differed between inbred and hybrid rice. The degree of flowering synchrony did not correlate with outcrossing rate. Whereas red rice biotype was the primary determinant of successful outcrossing with CL161, RH was the most critical factor for outcrossing with CLXL8. The evening temperature between 1,800 to 2,200 h

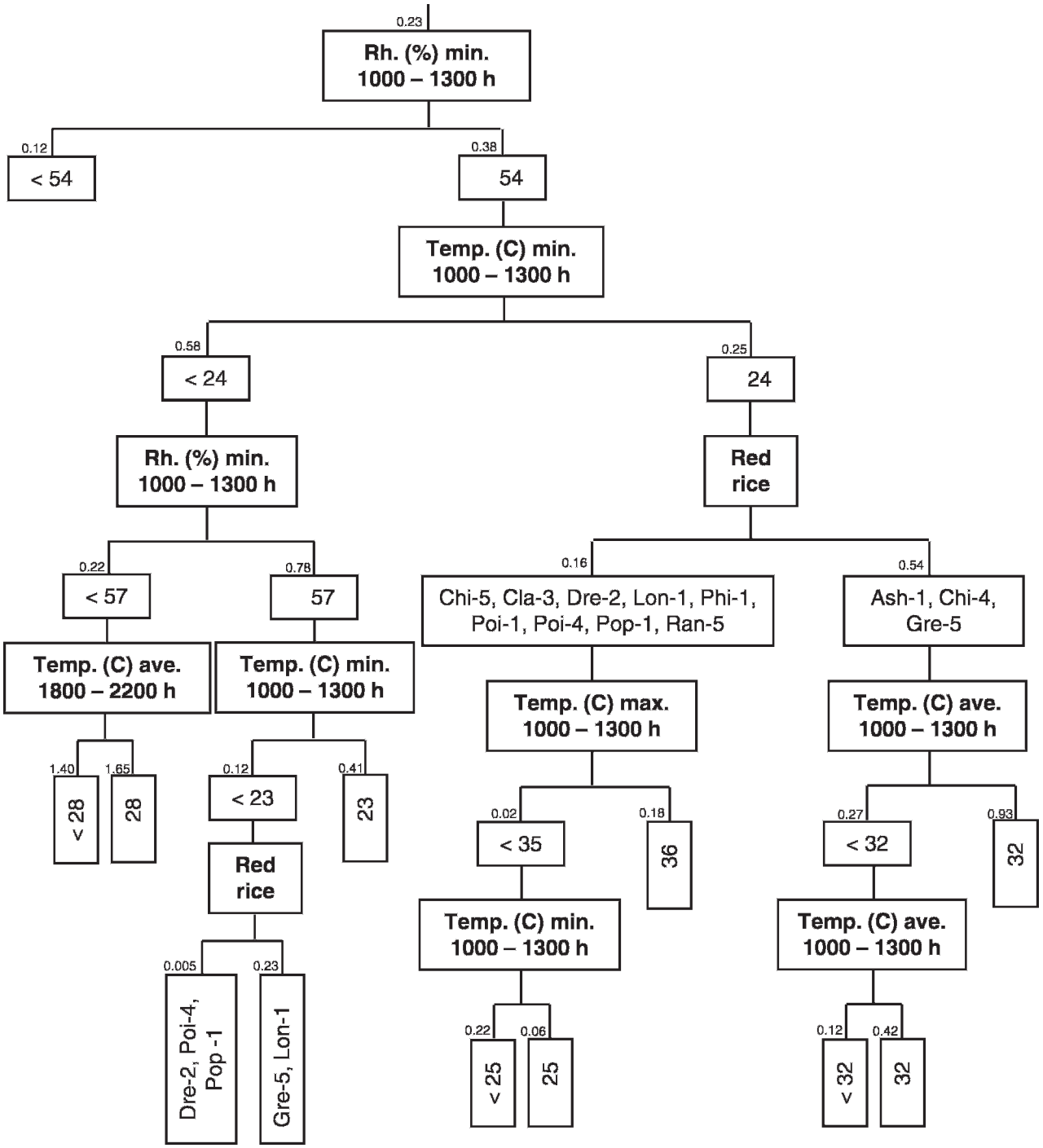


Figure 4. Partition flow diagram for the outcrossing rate of 12 red rice biotypes with CLXL8 rice. The relative importance of factors (in bold) decreases from top to bottom. Values at the nodes describe the average outcrossing rate of the subset of data that is evaluated at that partitioning. Abbreviations: Temp., air temperature; Rh., relative humidity; min., minimum; max., maximum; ave., average.

also strongly affected outcrossing with CL161. To clarify the interaction effects of these factors on outcrossing, experiments in controlled conditions with structured factor combinations need to be conducted. An expanded data set could then be used to construct models to predict the risk of outcrossing, given a set of variables. When planting HR rice, mitigate gene flow by characterizing, at least, the phenological trait of red rice biotypes in the field and implementing a combination of the following measures: (1) if possible, plant earlier or later to minimize the flowering synchrony between cultivated and weedy rice; (2) adopt the stale seedbed crop establishment method where the first batch of emerged red rice is killed by a nonselective herbicide, thereby reducing the infestation level and minimizing escapes from in-crop herbicide applications; (3) prevent escaped red rice from producing seed by spot spraying or rouging; (4) repeat the process in the next rice cropping seasons; and (5) rotate to another crop where weedy rice can also be controlled.

## Sources of Materials

[1] Delnet® bags, Delstar Technologies, Inc., 601 Industrial Drive, Middletown, DE 19709.

[2] HOBO model H8, Onset Computer Corporation, 470 MacArthur Boulevard, Bourne, MA 02532.

[3] Newpath Herbicide, BASF Agricultural Products, 26 Davis Dr., Research Triangle Park, NC 27709.

[4] Thermal cycler, Peltier-225 DNA Engine, MJ Research, Inc., Waltham, MA 02451.

[5] ABI 3730, Applied Biosystems Inc., 850 Lincoln Center Drive, Foster City, CA 94404.

[6] Genemapper, Applied Biosystems Inc., 850 Lincoln Center Drive, Foster City, CA 94404.

[7] SAS, Version 9.2, SAS Institute, Cary, NC 27513.

[8] JMP 8.0, SAS Institute, Cary, NC 27513.

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